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## Dysregulation of transcription and cytokine networks in Hodgkin lymphomas with a focus on nodular lymphocyte predominance type of Hodgkin lymphoma

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## **Summary and concluding remarks**

Lymph nodes involved by HL generally contain a minority of neoplastic cells surrounded by an abundant inflammatory infiltrate predominantly made up of CD4<sup>+</sup> T-cells, suggesting that immunological mechanisms contribute to HL pathogenesis. In this thesis, the transcriptional status and cytokine profile of the neoplastic and associated T-cells of HL were studied with particular emphasis on NLPHL.

### **NLPHL derived cell line DEV has a GC derived B-cell genotype and phenotype and an antigen presenting cell phenotype**

There is now overwhelming evidence indicating that the L&H cells of NLPHL have features of GC B-cells. DEV is the only cell line derived from NLPHL. In **Chapter 2** we have shown that DEV, similar to L&H cells, expresses B-cell-specific genes like CD79A, CD20, CD22 as well as molecules important for interaction with T<sub>H</sub> cells such as CD70, CD74, CD80 and CD86. Recurrent rearrangements of the *BCL6* gene have been detected in approximately half of the cases of NLPHL analyzed by FISH and by FICTION.(1;2) This suggests a critical role for *BCL6* in the development of NLPHL. We have demonstrated expression of *BCL6* protein and a break in the *BCL6* ABR region in DEV. In collaboration with the Institute of Human Genetics in Kiel, we did not detect any *BCL6* ABR breaks but did find *BCL6* MBR breaks in 2 of 12 NLPHL cases with FICTION analysis. Our findings corroborate the findings that in NLPHL *BCL6* rearrangements are relatively common.

### **T-cell TF phenotypes of HL associated T-cells are related to distinct lymphoid compartments**

NLPHL recapitulates lymphoid follicles as evidenced by morphology and immunophenotype. Neoplastic follicles contain a mix of GC B-cells, accessory T-cells, histiocytes, FDCs and many mantle zone B-cells. In **Chapter 3**, we describe that L&H cells are surrounded by an increased number of c-Maf<sup>+</sup>/CD57<sup>+</sup> T-cells, forming rosettes around the L&H cells. In transformed follicles of the PTGC cases there was also an increased frequency of c-Maf<sup>+</sup> T-cells. This finding supports the hypothesis that PTGC and NLPHL may be manifestations of an abnormal follicular centre reaction.

cHL is usually established in the interfollicular compartment of the lymph nodes. Indeed the T-cell TF profile of cHL associated T-cells indicates the same pattern as observed in the interfollicular compartment of reactive lymphoid tissues i.e. predominance of T-bet<sup>+</sup> T-cells and lesser amounts of GATA3<sup>+</sup> T-cells. The T-cell TF expression pattern of the HL associated T-cells was therefore remarkably consistent with the T-cell TF pattern observed in the distinct lymphoid compartments where HL subtypes are diagnosed. Our findings imply that the background T-cells of HL are not randomly distributed, but constitute an expansion of the native T-cells or an influx of T-cells which adapt to the same profile as the native T-cells in the corresponding lymphoid compartments.

### **Expression of the T-cell transcription factors in the neoplastic cells of HL: a transcriptional switch?**

In contrast to the previously reported deficiency of B-cell specific transcription factor (TF) expression in HL, (3-5) we observed frequent expression of the TFs GATA3 and T-bet in the neoplastic cells of HL and in HL cell lines, which may indicate a transcriptional switch in HL (**Chapter 4**).

HRS cells of cHL, express the TF NOTCH1, which is normally not expressed by B-cells and suppresses B-cell differentiation in lymphoid precursors in favour of T-cell differentiation.(6) Outcome of NOTCH1 activation in HRS cells may thus be a complete block of the B-cell developmental pathway with absence of Ig and B-cell specific antigens and aberrant expression of other haematopoietic lineage markers.(7;8) However, there are no data in the literature about the effect of NOTCH1 activation on the expression levels of T-cell TFs and the related cytokines in committed B-cells. Mathas *et al.* (9) reported overexpression of the helix-loop-helix proteins ABF1 and ID2 in HRS cells which antagonize the function of the B-cell determining TF E2A. This also could result in disruption of the B-cell specific TF program and in upregulation of genes that are normally not associated with the B-lineage.

Among the T-cell TFs expressed in the neoplastic cells of HL, GATA3 is generally known to be specific for CD4<sup>+</sup> T-cells. However, the master T<sub>H</sub>1 type TF T-bet is also expressed in normal and malignant B-cells, although its function in B-cells is not clear. Gerth *et al.* (10) described that T-bet can regulate the T-cell-independent

immune response. In a rodent model they demonstrated that T-bet is selectively required for IgG2a class switching, and plays an important role in protection against pathogens in response to T- cell independent stimuli, but also in humoral autoimmunity. Harris *et al.* (11) described a link between  $IFN\gamma$  and T-bet expression in B-cells. They showed that development of  $IFN\gamma$ -producing B-cells by either  $T_H1$  cells or IL12/IL18 is absolutely dependent on expression of  $IFN\gamma R$  and T-bet. They also demonstrated that although IL12/IL18 or  $IFN\gamma$ -producing  $T_H1$  cells are required to initiate transcription of the  $IFN\gamma$  gene in B-cells, sustained expression of  $IFN\gamma$  and T-bet by B-cells is dependent on an  $IFN\gamma/IFN\gamma R/T$ -bet autocrine feedback loop. The exact role of T-bet or the other T-cell TFs in the neoplastic cells of HL remains unknown.

### **The neoplastic cells of HL might be cytokine producing regulatory B-cells**

Several studies demonstrated that HL is associated with abnormal cytokine production (12;13). Since cytokine production is generally driven by expression of T-cell TFs, we analysed cytokine gene expression patterns of HL cell lines to determine the possible association with the target cytokine spectrum of the T-cell TFs. T-bet was expressed in 5 HL cell lines, and its cytokine target  $IFN\gamma$  was present in 4 of these, but none of them expressed  $IL2$ .  $GATA3$  was expressed in 4 cell lines and in L428 and L1236 the downstream targets of  $GATA3$ , being  $IL4$ ,  $IL5$  and  $IL13$  were indeed present. c-Maf was detected in 2 cell lines and  $IL4$  was indeed expressed in one of these. From our data in **Chapter 4**, we can draw the conclusion that the T-cell TF and the cytokine profiles of the cell lines show a significant degree of correlation. The presence of nuclear expression of T-cell TFs in the neoplastic cells of HL might thus explain the production of some specific cytokines in HL tissues and HL cell lines such as  $IL13$  (14) or  $IFN\gamma$ . However, overall the dominant cytokine expression profile of most of the HL cell lines including DEV is highly reminiscent of a  $CD4^+$  T-cell subset, designated as the  $T_R1$  subset, characterized by the ability to produce high levels of  $IL10$  and  $TGF\beta1$ .(15;16) As demonstrated previously for T-cells, recent studies also indicate the existence of regulatory B-cells.(17) These cells suppress immune mediated inflammations by mechanisms that include production of regulatory cytokines  $IL10$

and TGF $\beta$ 1, damping activated CD4<sup>+</sup> T-cells directly or by acting as a secondary APC. Based on the cytokine profiles of the HL cell lines, the anergic/immunoregulatory phenotype of HL associated T-cells (18;19) and the APC-like phenotype of the neoplastic cells of HL (20), it can be suggested that neoplastic cells of HL may function as regulatory B-cells.

### **The CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of NLPHL have T<sub>R</sub>1 type cytokine profile**

In **Chapter 5**, we demonstrated that the cytokine expression profile of CD4<sup>+</sup>/CD57<sup>+</sup> T-cells is consistent with a T<sub>R</sub>1 like immune response. Among the control T-cell subsets, CD4<sup>+</sup>/CD57<sup>+</sup> T-cells from tonsils were major producers of *IL4* mRNA. In contrast to these CD4<sup>+</sup>/CD57<sup>+</sup> T-cells from tonsils, *IL2* and *IL4* mRNA was consistently absent from the CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of NLPHL. Even after stimulation, no *IL4* transcripts could be detected in the CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of NLPHL. Abundant *IFN $\gamma$* , *IL10* and *TGF $\beta$ 1* expression in the absence of *IL2* or *IL4* suggested a T<sub>R</sub>1 immune response in the CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of NLPHL.(15) AID is indispensable for class switch recombination and somatic hypermutation of Ig genes. Expression of AID has been detected in GC centroblasts and in lymphomas derived from GC cells. In human B-cells, AID expression is induced by IL4 and enhanced by CD40 signalling.(21) Despite the lack of *IL4* mRNA and CD40L in the rosetting CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of NLPHL, L&H cells consistently express AID (22) and Ig (23). It can be speculated that L&H cells in spite of absence of T-cell help, gain GC B-cell features, however due to the absence of CD40L and/or IL4 signalling from the surrounding T<sub>R</sub>1 type T-cells further GC proliferation and/or plasma cell differentiation of L&H cells is precluded. Contrary to their strong c-Maf expression (**Chapter 3**), absence of *IL4* transcripts in CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of NLPHL is remarkable. This might suggest that in NLPHL, c-Maf induced expression of IL4 is blocked. c-Maf also has a transforming ability (24) and has been shown to function as an oncogene (25). Functional significance of c-Maf<sup>+</sup> T-cell rosettes in NLHPL cases remains to be elucidated.

### **T-cell TF and cytokine expression profile of the CD4<sup>+</sup>/CD57<sup>+</sup> T-cells of PTGC and NLPHL show similarities**

The cytokine analysis of the T-cells from PTGC cases was hampered by the low number and the heterogeneous composition of the PTGC cases, including both

PTGC and reactive GCs. Nevertheless the cytokine pattern of the transformed follicles of PTGC could be inferred from the differences between the cytokine profiles of tonsils and PTGCs. Cytokine profiles of the T-cells of the PTGC cases showed similarities as well as differences with tonsillar and NLPHL T-cells. Lower levels of *IL4* transcripts in  $CD4^+/CD57^+$  T-cells and higher levels of *IFN $\gamma$*  transcripts in all T-cell subsets of PTGC cases were similar to the results in NLPHL T-cells and most likely resulted from the T-cells in the transformed follicles. The similar T-cell TF (**Chapter 3**) and cytokine expression profile (**Chapter 5**) in the  $CD4^+/CD57^+$  T-cells of PTGC and NLPHL suggests that this feature is not the result of interaction with the L&H cells, but rather predisposes for the development of L&H cells.

### **cHL associated T-cells also have characteristics of regulatory T-cells**

Marshall *et al.* showed presence of IL10 secreting  $T_R1$  and  $CD4^+/CD25^+$  regulatory cells in cHL.(18) High numbers of FOXP3<sup>+</sup> T-cells were demonstrated in some cHL cases (26). We have shown that the reactive T-cells in cHL cases are predominantly immunoreactive for T-bet. Nevertheless, the TF expression repertoire of the majority of the reactive T-lymphocytes of cHL indicates a specific subpopulation of T-regulatory cells (27) with expression of T-bet, FOXP3, ICOS (Atayar *et al.*, unpublished results), *IL10* and *IFN $\gamma$* .

It has now become clear that the specific cellular environment in both types of HL-affected tissues is characterized by the presence of regulatory T-cells. The critical question is whether the regulatory T-cells in HL play a role in immune surveillance, thus maintaining the lymphoma 'in check' (especially considering the very favourable prognosis of NLPHL) or alternatively reflect a milieu required by the neoplastic cells of HL in order to survive. The regulatory type cytokine profile of the neoplastic cells of HL cells might help to create a uniquely favourable microenvironment protecting the neoplastic cells from cell-mediated apoptosis. On the other hand, the surrounding regulatory T-cells might also prevent the neoplastic cells to disseminate freely to the peripheral blood. At least, our studies indicate that to consider the HL associated T-cells as bystanders, as reactive cell components

#### *SUMMARY AND CONCLUSIONS*

or even as an immune host response to the neoplastic cells is a simplistic view. Signalling in HL is not unidirectional but bi-directional in both subtypes of HL.

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